CONSTRUCTION OF sgRNA EXPRESSION SYSTEM AGAINST BRCA1 GENE USING CRISPR-Cas9 TECHNOLOGY

Project report submitted to Sister Nivedita University in partial fulfilment of the requirement for the degree of

MASTER OF SCIENCE
IN
BIOTECHNOLOGY

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TO WHOM IT MAY CONCERN

This is to certify that Mr. **Arkaprava Dasadhikary**, a student of the Department of Biotechnology, Sister Nivedita University, has successfully completed his long-term training at **ATGC Diagnostics Pvt. Ltd., West Bengal, India**, in the domain of Molecular Biology, from **4th January 2025 to 16th May 2025.**

During this period, he was engaged in a research project titled **"Construction of sgRNA expression system against BRCA1 gene,"** under the supervision of **Dr. Susanta Roychoudhury**, Principal Investigator (PI) of the project. The project involved the design and development of a CRISPR-based sgRNA expression system targeting the BRCA1 gene.Mr. Dasadhikary was trained in and performed various core molecular biology techniques including restriction digestion, ligation, plasmid isolation, bacterial transformation, recombinant plasmid construction, and agarose gel electrophoresis. He also gained exposure to the principles of CRISPR-Cas9 genome editing, sgRNA design, vector selection, and expression validation.

Mr. Dasadhikary demonstrated a high level of enthusiasm, responsibility, and dedication throughout the training. His ability to grasp new techniques quickly and apply them effectively in the laboratory was commendable. He maintained professionalism and contributed positively to the lab environment, both individually and as a team member.

We appreciate his efforts and wish him continued success in his academic and professional pursuits.

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I hereby declare that the work which is being presented in this dissertation entitled **"Construction of sgRNA expression system against BRCA1 gene using CRISPR-Cas9 technology"** in partial fulfilment of the requirements for the award of the degree of Masters of Science in Biotechnology is an authentic record of my own work carried out during the period from **4th January,2025 to 16th May,2025** under the supervision of **Dr, Susanta Roychoudhury, PhD, FNA, FASc, FNASc, Director, ATGC Diagnostics Pvt Ltd.**

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ABBREVIATIONS

- µl – Microlitre
- ng – Nanogram
- µg – Microgram
- µM – Micromolar
- Amp – Ampicillin
- bp – Base pair
- BRCA1E10 – Breast Cancer gene 1 Exon 10
- DNA – Deoxyribonucleic acid
- DH5α – Douglas Hanahan's strain alpha
- dNTP – Deoxyribonucleotide Triphosphate
- *E. Coli* – Escherichia coli
- EcoRI – Escherichia coli R strain Y13
- EtBr – Ethidium bromide
- HDR – Homology-Directed Repair
- LA – Luria-Bertani Agar
- LB – Luria-Bertani broth
- ml – Millilitre
- NHEJ – Non-Homologous End Joining
- OD – Optical Density
- PAM – Protospacer Adjacent Motif
- PCR – Polymerase chain reaction
- Psi – Per square inch
- RNA – Ribo Nucleic Acid
- rpm – Revolution per minute
- RT – Room temperature
- sg – Single Guide
- TAE – Tris acetate EDTA
- UV – Ultraviolet

ABSTRACT

The BRCA1 gene plays an important role in maintaining the genomic stability and mutation of this gene increases the susceptibility towards breast and ovarian cancers. Targeted genome editing technologies like CRISPR-Cas9 have revolutionized genetic research through enabling precise genomic manipulation. The construction of a single -guide RNA (sgRNA) expression system targeting the BRCA1 gene represents a fundamental step towards elucidating gene function, modelling diseases and potentially developing gene therapies. In this study we have designed and constructed an sgRNA expression system specifically targeting 10^{th} exon of the BRCA1 gene. Bioinformatic tools like NCBI, BLAST and CHOPCHOP were used to identify optimal sgRNA target sites with minimal off-target potential. The designed sgRNA was then cloned into a compatible expression vector, pSpCas9(BB)-2A-Puro (PX459). After successfully cloning the recombinant clones were confirmed through end-point PCR and Sanger's sequencing. This research highlights the accuracy and feasibility of constructing sgRNA expression system targeting the BRCA1 gene and provides foundation for functional genomics studies.

Keywords: BRCA1, CRISPR-Cas9, Gene editing, Restriction Digestion, Ligation, NCBI, Cloning, PX459, End point PCR, Sanger's Sequencing

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INTRODUCTION

The Breast Cancer 1 gene or BRCA 1 gene is a crucial component of human genome which plays an important role in maintaining genomic stability. It is located on chromosome 17 at position q21 and consists 24 exons. BRCA1 codes for a tumor suppressor protein which plays crucial role for DNA damage repair and also maintains other cellular process. BRCA1 along with other repair proteins forms a multi-protein complex known as the BRCA1-associated genome surveillance complex or BASC, which operates the repair mechanism of double-strand DNA breaks (DSB) in a highly accurate process called Homologous Recombination (HR). In case of a normal human when a DNA damage occurs, BRCA1 gets recruited to the break site where it helps to recognize the damage and facilitates end resection by creating single-stranded DNA overhangs. It then assists in recruiting and stabilizing RAD51 which is a key recombinase and uses a sister chromatid as a template and accurately repairs the damaged DNA. Through this BRCA1 maintains the genomic stability and prevents the accumulation of genetic mutation that can potentially lead to uncontrolled cell division and cancer. But in case of inherited or acquired mutations in a BRCA1 gene, it loses its ability to participate in Homologous Recombination. This loss of function in BRCA1 leads the cells with dysfunctional BRCA1 gene to use error-prone DNA repair pathways such as non-homologous end joining (NHEJ) which frequently introduces mutations at DNA breakage sites. These mutations get accumulated in the cells and overtime, this accumulation of genetic alterations disrupts other critical genes like oncogenes and tumor suppressors, which leads to uncontrolled cell division and tumor development ultimately leading to cancer. BRCA1-related cancers often exhibit aggressive clinical features, including early onset and poor differentiation and women with BRCA1 mutation have a 65% lifetime risk of developing breast cancer and 39% risk of ovarian cancer *(Cold Spring Harb Perspect Biol. 2015 Apr;7(4):a016600. doi: 10.1101/cshperspect.a016600).*

CRISPR-Cas9 technology, a novel and robust genome editing tool can be strategically employed to overcome these fatal effects of BRCA1 mutations by selectively knocking out mutated alleles which produces a dysfunctional BRCA1 protein. Though in nature it acts as a bacterial adaptive immunity response system, the Clustered Regularly Interspaced Short Palindromic Repeats or the CRISPR-Cas9 system is an RNA mediated system which is now used by the scientists to guide the Cas9 endonuclease to target DNA site and generate indels.

For an effective CRISPR mediated knockout the sgRNA has to be effectively designed as it controls the specificity, efficiency and outcome of the CRISPR mechanism. The single guide RNA (sgRNA) has two domains fused together, CRISPR RNA (crRNA) a 20-nucleotide sequence and the trans-activating CRISPR RNA (tracrRNA). This single RNA molecule binds with the Cas9 endonuclease and guides it to the precise target DNA sequence located immediately upstream of a PAM (Protospacer Adjacent Motif) sequence. Cas9 binds to the target sequence and it induces a double-strand break at the target site. The cell then repairs the break, through non-homologous end joining (NHEJ) which often introduces insertions or deletions (indels), that disrupts the reading frame and effectively inactivating the mutant allele. Enabling gene disruption, repair or replacement of the mutated gene. *(Cureus. 2024 Jul 11;16(7):e64324. doi: 10.7759/cureus.64324).*

This dissertation project focuses on designing an effective sgRNA targeting the 10th exon of the BRCA1 gene which allows for precise CRISPR-Cas9 mediated knockout of this specific exon. The sgRNA was designed using bioinformatic tools like NCBI, BLAST and CHOPCHOP and then cloned into compatible expression vector, pSpCas9(BB)-2A-Puro (PX459). The 10th exon of BRCA1 encodes parts of the gene involved in DNA repair, by selectively knocking out this exon, the faulty gene transcript maybe degraded via nonsense-mediated decay, reducing the production of a dysfunctional protein. This strategy can then be paired with different gene correction or compensatory therapy. Hypothetically, the outcome of this experiment would be the reduction of aberrant BRCA1 activity, restoration of some DNA repair capability and increased cancer cell death due to therapy sensitivity. (*Witz, A., Dardare, J., Francois, A. et al. CRISPR/Cas9-mediated knock-in of BRCA1/2 mutations restores response to olaparib in pancreatic cancer cell lines. Sci Rep* ***13****, 18741 (2023). https://doi.org/10.1038/s41598-023-45964-w).*

This approach of targeted gene editing holds the potential for personalized gene therapy and carries the potency of being used in clinical applications after further standardizations.

REVIEW OF LITERATURE

BRCA1 is an important tumor gene that encodes a multifunctional protein essential for replication fork stability, transcriptional regulation and for DNA Damage repair. The N-terminal RING domain of BRCA1 facilitates E3 ubiquitin ligase activity through its interaction with BARD1 and the C-terminal BRCT domain mediates phosphoprotein interactions important for homologous recombination (HR). Pathogenic mutations in these functional domains of BRCA1 impairs HR-mediated repairs of DSBs, leading to genomic instability and increases risk of breast, ovarian and other cancers *(Front Oncol. 2025 Feb 6;15:1497531. doi: 10.3389/fonc.2025.1497531).*

Thus, the accurate identification and functional assessment of BRCA1 variants are essential for clinical management, therapeutic decision making and risk assessment. With the advent of CRISPR-Cas9 mediated genome editing technology, precise genome editing has become a powerful tool for studying gene function and developing novel therapeutic strategies for BRCA1 gene. This novel technology employs a guide RNA (sgRNA) which guides the Cas9 nuclease to a specific genomic locus, resulting in DSBs which can be repaired by either NHEJ or HDR *(Nature. 2018 Sep 12;562(7726):217–222. doi: 10.1038/s41586-018-0461-z).* CRISPR-Cas9 based have strategies been used to model BRCA1 mutations in different cell-lines to assess variant pathogenicity and study essentiality of the gene (*Oncogene **39**, 30–35 (2020). https://doi.org/10.1038/s41388-019-0968-2).*

The 10th exon of BRCA1 is the largest exon of the gene, but it does not encode any clinically significant residues or functional protein domains directly associated with the RING or BRCT domains. Despite this, any structural or regulatory alterations in exon 10 can have profound effects on BRCA1 stability and its different cellular activities. Recent studies suggest that novel partial duplications and rearrangements in BRCA1 exon 10 can lead to premature stop codon or disrupt the reading frame which results in non-functional BRCA1 proteins. Thus, to understand detailed molecular characterization and clinical significance we need robust experimental systems to assess the impact *(Front. Cell Dev. Biol., 01 March 2022 Sec. Signaling, Volume 10 - 2022| https://doi.org/10.3389/fcell.2022.813457).*

The construction of sgRNA expression system targeting BRCA1 exon 10 is a logical extension of pre-existing CRISPR-Cas9 methodologies. sgRNA designed to target specific region within

exon 10, enables precise editing and functional assessment of this large and complex exon. Using a plasmid vector (PX459) as sgRNA delivery system allows for a stable integration and persistent expression in target cells, enabling long-term studies of BRCA1 function and effects of exon modifications *(Oncogene **39**, 30–35 (2020). https://doi.org/10.1038/s41388-019-0968-2)*.

Through constructing an sgRNA expression system targeting the 10th exon of BRCA1 gene, this thesis aims to establish a versatile experimental platform for studying the functional consequences of exon 10 alterations. This approach will contribute in accurately classifying BRCA1 variants, improve our understanding of exon 10 and BRCA1 biology, and overall, the integration of CRISPR-Cas9 technology with sgRNA expression system will support the development of targeted therapies for BRCA1 associated cancers.

MATERIALS

Standard good lab practices and routine aseptic conditions were followed while performing all experiments. All lab wares (flasks, beakers, tubes, plates), media, reagents, disposable wares (tips, tubes, loops, spreaders) were sterilised by autoclaving at 121˚C/15 psi for 15 minutes.

Plasmid–pSpCas9(BB)-2A-Puro(PX459)[https://www.addgene.org/browse/sequence/456946] was provided to us by Ms. Anupa Chattopadhyay (Saroj Gupta Cancer Research Centre), Oligomers (Ordered from Syngynx) *E.coli* strain DH5α, Ampicillin (100mg/ml), LB medium (SRL), Agar-agar (SRL), 10X TAE Buffer, Ethidium Bromide, EcoRI Restriction Endonuclease (5units/µl), BbsI Restriction Endonuclease (10units/µl), Ligase, Taq Polymerase, DNTP, 10X PCR Buffer, NFW

METHODS

1. sgRNA Designing and Cas9 Vector Construction

Primarily we need to design an efficient single guide RNA for targeting exon 10 of BRCA1 gene with the Px459 Cas 9 vector. The sgRNA designing is an important step for CRISPR-Cas9 mediated knockout. In order to do that an online software CHOPCHOP (https://chopchop.cbu.uib.no) was used and the sgRNA sequences were generated by us following the steps mentioned below.

Firstly, NCBI (https://www.ncbi.nlm.nih.gov) portal was opened. Next, BRCA1 gene was searched. Next, in 'GenBank' option we searched and selected our desired Transcript Id (NM_007294.4) of the BRCA1 gene. After selecting our desired Transcript Id, our desired exon (Exon 10) was searched and the sequence was copied. Next, the copied exon sequence was pasted in CHOPCHOP and specific parameters for the designed sgRNA were set.

For 'Target specific region of the gene' option 'Coding Region' was selected. For 'Restrict targeting' option 'Search exons and Immediate short flanking regions' was selected. In the 'Isoform consensus determined by' option 'Intersection' option was selected. In 'Pre-filtering' option the 'Minimum required GC (%) content' was set between Minimum: 40% and Maximum:60%.

Based on our given parameters the software generated multiple sequences. After comparing the efficiency, GC content, self-complementarity and other parameters our desired top and bottom sgRNA sequences were selected and ordered from Syngynx.

Target Name	Vector	Guide RNA Sequence
BRCA1 coding. E10 guide Top Strand	PX459	5' CACCGGGAGTTGGTCTGAGTGACA 3'
BRCA1 coding. E10 guide Bottom Strand	PX459	5' AAACTGTCACTCAGACCAACTCCC 3'

Table 4.1.1 – Target exons and designed primer sequences.

2. Annealing of Oligomers

After the designed sgRNAs were delivered to us we needed to anneal the oligomers in order use it in our downstream experiments. To anneal our oligomers first, we ran an empty spin to collect the lyophilized oligomers in the bottom of the test vials. Next, nuclease free water was added in the vials to dilute the lyophilized oligomers. 308.5µl of nuclease free water was added to the top strand oligomer and 313.5µl of nuclease free water was added to the bottom strand of the oligomer to attain 100Mm concentration and another quick spin was performed for proper mixing. Next a reaction mixture was set up by using the components mentioned below in (Table 4.2.1).

Components Name	Volume
Top oligomer (100µM)	2.5 µl
Bottom oligomer (100µM)	2.5 µl
10x PCR (Taq) buffer	5 µl
Nuclease free water	40 µl
Total Reaction volume	50 µl

Table 4.2.1– Reaction mixture components and volume for annealing of oligomers.

The reaction mixture was then subjected to thermal cycling in a PCR machine with the reaction steps, temperature and time mentioned in (Table 4.2.2).

Step No.	Temperature (^{0}C)	Time (minutes)
1	95	2
2	90	5
3	85	5
4	80	5
5	75	5
6	70	5
7	65	5
8	60	5
9	55	5
10	50	5
11	45	5
12	40	5
13	35	5
14	25	5
15	4	∞

Table 4.2.2 - Thermal cycler steps, temperature and time for annealing of oligomers.

3. Phosphorylation of Annealed Oligomers

After successfully annealing, the annealed oligomers were needed to be phosphorylated for our following downstream process. For this the double stranded oligomers were subjected to 5' phosphorylation by incubating our reaction mixture at 37˚C for 2 hours. A reaction mixture was setup using the components mentioned below in (Table 4.3.1).

Name of Components	Volume
Annealed Oligomer	44 µl
T4 polynucleotide kinase (PNK) (10 units/µl)	1 µl
10x PNK buffer	5 µl
Total Reaction volume	50 µl

Table 4.3.1 - Reaction mixture components and volume for phosphorylation of oligomers

4. DH5α Cell Culture Preparation

We prepared the primary cell culture of DH5α cells by first pouring 5ml volume of LB broth in a 50 ml conical centrifuge tube. Next by using a single pipette tip we isolated a single colony of DH5α from the host culture plate and dropped the tip ensuring that the tip is completely submerged into the LB broth. Next, we put the centrifuge tubes in the shaker-incubator to incubate overnight at 37˚C and 130 rpm. After 16hrs incubation, the primary culture was removed from the shaker-incubator. Next the secondary culture was prepared through mixing 5ml volume of LB broth along with 500µl volume of the primary culture. This preparation was then incubated at 37˚C and 230 rpm until the O.D. of the preparation attained 0.4-0.6 at 600nm wavelength. The O.D. was confirmed using Spectrophotometer.

5. Competent DH5α Cells Preparation

The total 5ml volume of DH5α secondary culture was aliquoted into 1.5 ml microcentrifuge tubes. Next, the microcentrifuge tubes were centrifuged at 10,000 rpm for 1 minute at room temperature. The supernatants were then discarded completely using a 20-200µl pipette. Next, we resuspended the cell pellets in 600µl volume of pre-chilled transformation buffer (pH 7.15). The whole solution was then incubated in ice for 15 minutes. Once the incubation completed, the resuspended cells were again centrifuged at 10,000 rpm for 1 minute at room temperature. The supernatants were then discarded completely and the cell pellets were resuspended in 200µl volume of pre-chilled sterile transformation buffer. This cell suspension contains

competent DH5α cells which were then divided into 2 sets- Tp and Tn, each set consisted 2 microcentrifuge tubes and next they were subjected to transformation.

6. Transformation of Competent DH5α Cells

After competent cell preparation, the competent DH5αcells were divided into 2 sets:

In the 'Tp' Set, 2µl volume (100ng/µl) of PX459 plasmid was added, and the 'Tn' set consisted only competent DH5α cells. These set division was done to ensure that no unwanted contaminants were present. The tubes of both sets were incubated in ice for 10 minutes. Next, the tubes were transferred to a pre-heated water bath for 90 seconds, with the temperature of the water bath set at 42˚C for heat shock. The tubes were then taken out of the water bath and rapidly cooled on ice and incubated for 5 minutes. Next, 800µl volume of LB broth was added to all the tubes and incubated for 1-1.5 hours in the shaker incubator at 37˚C and 150 rpm. Once the incubation completed, 200µl volume of cell culture from each tube was plated on LB agar + ampicillin plate and incubated at 37˚°C overnight. After 16 hours of incubation, the plates were then removed from the incubator, and the bacterial growth was observed. As the PX459 plasmid has ampicillin resistant gene, growth of bacterial colonies was observed in the 'Tp' plates, while colonies were absent in the 'Tn' plate. Next, we isolated a single colony from the 'Tp' plate and replica plated it on a LB agar + ampicillin plate and incubated overnight at 37˚C.

7. Plasmid DNA Isolation of Transformed Cells

After a successful transformation of the plasmid into the bacterial cells, a volume of 10 ml of LB + ampicillin growth medium was prepared in two separate 50 ml centrifuge tubes. Next, a single colony was isolated for each tube from the replica plate and added to our LB + ampicillin culture. This culture was then put in the shaker-incubator for bacterial growth at 37˚C and 130 rpm. After 16 hours of incubation, the tubes were taken out of the shaker-incubator, and 4.5 ml volume of the cell culture was aliquoted into three microcentrifuge tubes (1.5 ml each) and centrifuged at 1000 rpm for 3 minutes. After the first centrifugation, the supernatant was discarded carefully using a micropipette. The remaining 4.5 ml volume of the cell culture was then aliquoted into the same centrifuge tubes and centrifuged again for 3 minutes at 10000 rpm.

The supernatants were then discarded, and the cell pellets were resuspended by adding 250µl of P1 buffer (Resuspension buffer). The mixture was aspirated carefully until it turned cloudy with no visible cell clumps. Next, 250µl volume of P2 buffer (Lysis buffer) solution was added into each tube and was gently inverted mixed for 2 minutes. Immediately after 2 minutes of inverted mixing, 350µl of PN-3 buffer (Neutralizing buffer) was added to the solution, and the solution was gently inverted and mixed for 2-3 minutes. Next, the tubes were centrifuged at 10000 rpm for 10 minutes. Two distinct layers were noticed inside the microcentrifuge tube at this stage. The clear supernatant was separated from three tubes and added to a single spin column using a micropipette and centrifuged at 10000 rpm for 1 minute. Once the centrifugation was done, the flowthrough was discarded. This step was repeated until the entire supernatant from three tubes was spun down in a single spin column. After this, we added 500µl of PW-1 wash buffer solution into the spin column and centrifuged the spin column for 1 minute at 10000 rpm. The flowthrough was discarded, and 700µl of PW-2 wash buffer solution was added into the spin column and centrifuged for 1 minute at 10000 rpm. The flowthrough was discarded, and an additional empty centrifugation was done at 10000 rpm for 1 minute to exclude any excessive wash buffer present. Next, we placed the spin column in a 1.5ml microcentrifuge tube and added 50µl pre-warmed distilled water as our elution medium. The addition was done in two steps, where we first added 30µl of the pre-warmed distilled water and incubated for 2 minutes, and then centrifuged it for 1 minute at 10000 rpm, and next we added the rest 20µl of distilled water and repeated the same centrifugation protocol. Our yield after completion of this process was 50µl of plasmid DNA. Next, we checked its concentration using nanodrop spectrophotometer (249.4 ng/µl) and ran it in 1% agarose gel along with 1kb DNA ladder for size confirmation and visualized it under UV.

8. Agarose Gel Electrophoresis:

In order to confirm the size of DNA we ran the DNA in agarose gel as a confirmatory step after several experiments required for this project work. In order to do the experiment first we prepared 1% gel by mixing 0.3g of agarose was added in 30ml TAE buffer in a conical flask. Next, the mixture was put in a microwave and heated until the agarose was completely dissolved into the TAE buffer. The gel tray was set up during this time, the combs were placed and the sides of the gel tray was sealed using tape to prevent any leakage. After the agarose is

completely dissolved in the mixture was cooled under running tap water and 2µl EtBr was added and mixed. The liquid mixture was then poured into the gel tray and allowed to solidify. After 10-15 minutes after the gel solidifies the combs were removed and the chamber was filled with 1X TAE buffer until the gel was completely submerged. Next, 1X gel loading dye was mixed with DNA and mixed well and then loaded into the gel. 1kb DNA ladder was also loaded along with the DNA sample. Finally, electrophoresis was performed at different voltages and time required for different experiments.

9. EcoRI Restriction Digestion Confirmation of PX459 Plasmid

This was a confirmatory step to ensure that the isolated plasmid DNA was indeed PX459. For this test we took a 100µl microcentrifugation tube and prepared a reaction mixture using the components given below in (Table 4.9.1). The reaction mixture was incubated for 2 hours in the water-bath set at a temperature at 37˚C. After 2 hours incubation we visualized the DNA sample using 1.5% agarose gel and by compared the fragment size with a 1kb ladder and an undigested DNA sample of PX459 plasmid.

Name of Components	Volume
PX459 Plasmid DNA (249.4 ng/µl)	2 µl
EcoR1 enzyme (5 unit/µl)	0.5 µl
10x Buffer	2 µl
Nuclease free water	16 µl
Total Reaction volume	20 µl

Table 4.9.1 - Reaction mixture components and volume for EcoRI restriction digestion.

10. BbsI restriction digestion of Isolated PX459 Plasmid

After confirming presence of PX459 plasmid through EcoRI restriction digestion, we treated our plasmid with BbsI restriction endonuclease. The reaction mixture was set up by using the components mentioned below in (Table 4.10.1).

Name of Components	Volume
Plasmid PX459 (249.4 ng/µl)	8.05 µl
BbsI enzyme (10 unit/µl)	1 µl
10x BbsI buffer	5 µl
Nuclease free water	36 µl
Total Reaction volume	50 µl

Table 4.10.1 - Reaction mixture components and volume for BbsI restriction digestion.

This reaction mixture was incubated for 2 hours in the water-bath set at a temperature of 37˚C. We visualized the DNA sample using 1.5% agarose gel and by comparing the fragment size with a 1kb ladder and an undigested DNA sample we confirmed the restriction digestion. After this in order to remove any unwanted restriction enzyme present, we purified the BbsI Digested DNA using SLS PCR purification kit. For purification we first aliquoted our Bbs1 digested DNA into a spin column and added PW1 buffer in it. Then we centrifuged the column at 10,000 rpm for 1 minute and discarded the flowthrough. Next, we added PW2 into the spin column and again centrifuged the column at 10,000 rpm for 1 minute and discarded the flowthrough and performed an empty spin to eliminate any excess wash buffer. Finally, we eluted our purified PX459 using 50µl pre-heated nuclease free water.

11.Ligation of sgRNA in PX459 Plasmid

After purifying our digested plasmid DNA in order to ligate our designed annealed sgRNA oligomers into the plasmid DNA we setup two sets of ligation reaction named L1 and L2. The L1 set was incubated at 16˚C for overnight and the L2 set was incubated at room temperature for 6 hours. The reaction mixture was prepared in a 100µl microcentrifuge tube using the components mentioned below in (Table 4.11.1) and (Table 4.11.2). A negative control was also set up which contained all the mentioned components except the annealed phosphorylated oligomers.

Name of Components	Volume
Vector DNA (16 ng/µl)	4 µl
Insert DNA (1:100 dilution)	1 µl
T4 Ligase	1 µl
10X Ligation Buffer	1 µl
Nuclease free water	4 µl
Total Reaction volume	11 µl

Table 4.11.1 - Reaction mixture components and volume for 'L1' set of sgRNA ligation in PX459 plasmid.

Name of Components	Volume
Vector DNA (16ng/µl)	10 µl
Insert DNA (1:100 dilution)	2 µl
T4 Ligase	0.5 µl
10X Ligation Buffer	2 µl
Nuclease free water	5.5 µl
Total Reaction volume	20 µl

Table 4.11.2 - Reaction mixture components and volume for 'L2' set of sgRNA ligation in PX459 plasmid.

12. Transformation and Replica Plating of Competent DH5α Cells

After ligation of the vector and insert, we transformed it into competent DH5α cells following the same standardised protocol for transformation we followed previously. After a successful transformation of the ligated plasmid, we isolated four colonies from the L1 and L2 sets and then resuspended each colony in 10µl of nuclease free water. From the 10µl of the resuspended colonies we replica plated 5µl in a checkerboard manner using inoculation loops and the remaining 5µl was used for colony PCR confirmation process.

13.Colony PCR Confirmation of PX459 Ligated DH5α Colonies

This was a confirmation step to ensure the proper ligation of our designed guide RNA into our plasmid vector PX459. We set up our reaction mixture (Table 4.13.1) using an insert specific primer (BRCA1 bottom oligomer) for 10 PCR tubes with 20 µl reaction mixture volume for each tube with the reaction components mentioned below. Next, we added 5µl of each resuspended colonies in separate PCR tubes and ran a colony PCR (Table 4.13.2). After the PCR we confirmed the ligation by running the PCR mixture in 1.5% agarose gel.

Name of Components	Volume
Taq Polymerase	2.5 µl
10x PCR buffer	20 µl
dNTP	20 µl
U6 Forward Primer (10µM)	5 µl
BRCA1 Bottom oligomer (10µM)	5 µl
Nuclease Free Water	147.6 µl
Total Volume	200 µl

Table 4.13.1 - Reaction mixture components and volume for Colony PCR of ligated DH5α colonies.

Stage	Temperature (^{0}C)	Time
1	98	5’
2	94	5’
3 (35 cycles)	94	30’’
	60	30’’
	72	40’’
4	72	5’
5	4	∞

Table 4.13.2 - Colony PCR cycles, temperature and time for each cycle.

After confirmation we isolated plasmid DNA from our ligated colonies using our standardized plasmid DNA isolation protocol and measured the concentration of isolated DNA using nanodrop spectrophotometer. Plasmid DNA from two recombinant clones with highest DNA concentration BRCA1ADE10.1 clone (176.4 ng/µl) and BRCA1ADE10.2 clone (104.7 ng/µl) were used to continue the downstream experiments.

14. Designing an Insert Specific Reverse Primer

For further confirmation of the ligation, we also designed a 18-25 nucleotide long vector specific reverse primer to run an end point PCR simultaneous to our insert specific primer end point PCR for comparison. For designing the reverse primer, first we opened SnapGene software (https://www.snapgene.com) and copied FASTA sequence 150bp away upstream and downstream from the BbsI cut sites. Next, we opened NCBI Primer BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primerblast) set parameters (Tm, GC Content and minimal secondary structures) and pasted the FASTA sequence. The NCBI Primer BLAST provided multiple primer sequence from which we selected the most compatible primer (Table 4.14.1) and ordered it.

U6 Forward Primer Sequence	5' GCCTATTTCCCATGATTCC 3'
Vector Specific Reverse Primer Sequence	5' AAACACCAGTTCTGCTCCATGAAGC 3'

Table 4.14.1 - U6 forward primer and designed vector specific reverse primer sequences.

15. PCR Comparison of Insert and Vector Specific Primer

After designing our vector specific we set up two PCR reaction mixture 'P1' and 'P2' where 'P1' contained the insert specific primer and the 'P2' contained the vector specific primer. We set our reaction with the reaction components mentioned below and ran end point PCR following the cycles mentioned below (Table 4.15.3).

After completion of the end point PCR cycles, we compared both amplicon sizes by running 1.5% agarose gel.

Name of Components	Volume
Ligated DNA (176.4 ng/µl)	1µl
Taq Polymerase	1 µl
10x PCR buffer	8 µl
dNTP	8 µl
U6 Forward Primer (10µM)	2 µl
BRCA1 Bottom oligomer (10µM)	2 µl
Nuclease Free Water	59 µl
Total Volume	80 µl

Table 4.15.1 - Reaction mixture components and volume for 'P1' set of end-point PCR.

Name of Components	Volume
Ligated DNA (176.4 ng/µl)	1µl
Taq Polymerase	1 µl
10x PCR buffer	8 µl
dNTP	8 µl
U6 Forward Primer (10µM)	2 µl
U6 Reverse Primer (10µM)	2 µl
Nuclease Free Water	59 µl
Total Volume	80 µl

Table 4.15.2 - Reaction mixture components and volume for 'P2' set of end-point PCR.

Stage	Temperature (^{0}C)	Time
1	98	5’
2	94	5’
3 (35 cycles)	94	30’’
	60	30’’
	72	40’’
4	72	5’
5	4	∞

Table 4.15.3 - Endpoint PCR cycles, temperature and time for each cycle.

16.Sanger’s Sequencing Confirmation of Recombinant Clones

After confirming a successful ligation of our designed guide RNA into our recombinant clones, we sequenced our recombinant clones through Sanger’s Sequencing method and located our sgRNA cloned in the vector plasmid PX459. We aliquoted 20 µl of plasmid DNA (2µg) into a 1.5 ml microcentrifuge tube for both recombinant plasmids BRCA1ADE10.1 and BRCA1ADE10.2. Next, we added 10 µl of U6 forward primer (10µM) into the 1.5 ml and 10 µl BRCA1 Bottom oligomer (10µM) in a microcentrifuge tube. Both aliquoted primers and plasmid DNA was sent at ‘Barcode’ for Sanger’s Sequencing. After sequencing the electropherogram of both of the plasmids were analysed carefully.

RESULTS & DISCUSSION

1. sgRNA modifications, annealing and phosphorylation

After selecting the most compatible sgRNAs we added – CAAC – in the 5' end of the top oligomer strand and – AAAC – in the 5' end of the bottom oligomer strand to create overhangs which would be necessary in our downstream processes. Once the sgRNAs were delivered to us we annealed the oligomers which involved thermal cycling of crRNA and tracrRNA parts of our designed sgRNA in a PCR machine and then allowing them to down slowly. This controlled change in temperature facilitates the hybridization of the complementary sequences within the sgRNA molecule which leads to the formation of a specific structure with defined stem loops and a double stranded region which is recognised by the Cas9 enzyme. The annealed oligomers were then phosphorylated following our protocol. The annealed oligomers were then phosphorylated which added a phosphate group to the 5' end of the oligomers which was necessary for our downstream experimental steps. After completion of these steps our functional sgRNA was ready to be inserted into the PX459 plasmid.

2. Plating Transformed DH5α Competent Cells

We used calcium chloride to prepare the DH5α competent cells as it increases the porosity of the cell membrane making it easier for foreign DNA uptake. In order to avoid 'false colonies' and make sure there is no contaminants present, we divided the competent cells into two sets - 'Tp' consisting competent DH5α cells + Plasmid PX459 which was our 'positive control' plate and 'Tn' consisting only competent DH5α cells which was our negative control plate. Next, we transformed our competent DH5α cells via heat-shock method which incorporated our PX459 plasmid DNA into the DH5α genome. Then we plated both sets on LB + ampicillin agar plate and incubated both plates overnight providing optimal conditions for growth. The plasmid PX459 that transformed into the competent DH5α consists ampicillin resistant gene, so after transformation we spread plated the transformed cells on LB + ampicillin agar plate. Ampicillin acts as a resistant marker and thus only the transformed DH5α cells are able to grow on the

agar plate. After overnight incubation we observed multiple colony growth on ‘Tp’ plate and no colonies on ‘Tn’ plate, confirming a successful transformation of PX459 plasmid into competent DH5α cells.

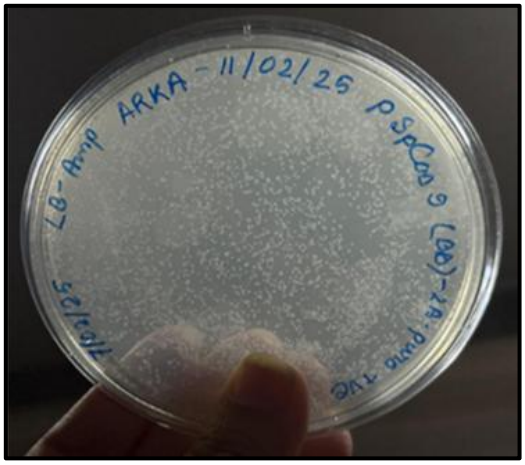

Fig.01 ‘Tp’ Set of Transformed DH5α

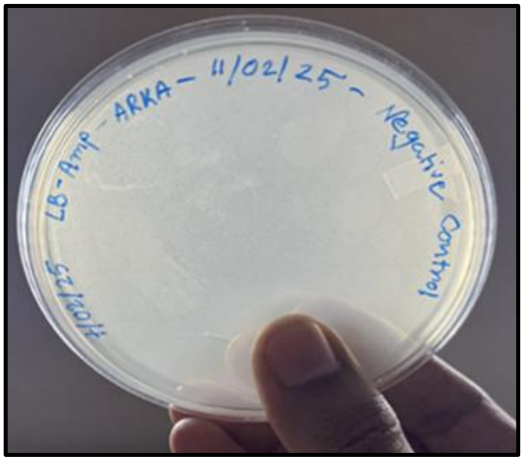

Fig.02 ‘Tn’ Set of Transformed DH5α

3. Single Colony Isolation and Plating

From the ‘Tp’ plate of transformed DH5α cells, we inoculated a single colony using an inoculation loop and streak plated it on a LB + ampicillin agar plate and incubated overnight. Ampicillin acted as a selection marker ensuring only the colonies with antibiotic resistant gene will be able to grow on the plate. This step was done to maintain purity for all our downstream experiments and help us troubleshooting if any contamination occurs. As the colonies we got on the streak plate after overnight incubation, grew from a single colony isolated from the ‘Tp’ plate their genotype is expected to be same and contain our plasmid DNA.

Fig.03 Replica Plated Single Colony.

4. Plasmid DNA Isolation and Confirmation

To confirm the isolated and replica plated single colony contained PX459 plasmid, we isolated plasmid DNA from the replica plated colonies following our standardized plasmid DNA isolation protocol and measured its concentration and presence of RNA contamination under a nanodrop spectrophotometer. After confirming its concentration (249.5 ng/µl) and checking its purity (A260/A280 = 1.81), we ran the DNA in 1% Agarose gel for confirmation of our plasmid DNA based on its size. The PX459 plasmid contains 9174 bp so along with the plasmid DNA we ran a 1kb DNA ladder for size comparison. We followed the standard protocol of Agarose gel electrophoresis and ran the experiment. After the electrophoresis completed, we visualized our gel and analysed the image. We found three distinct forms of plasmid DNA in our image. The very top band being the nicked form, the second band being the linear form and the third being the supercoiled form. We compared these bands with the DNA ladder and found our plasmid DNA band (supercoiled band) at 9.2 kb, which confirmed the presence of our plasmid DNA. The spectrophotometric analysis of the isolated plasmid DNA suggests it to be high quality and suitable for our downstream experiments like restriction digestion, Cloning, PCR and sequencing.

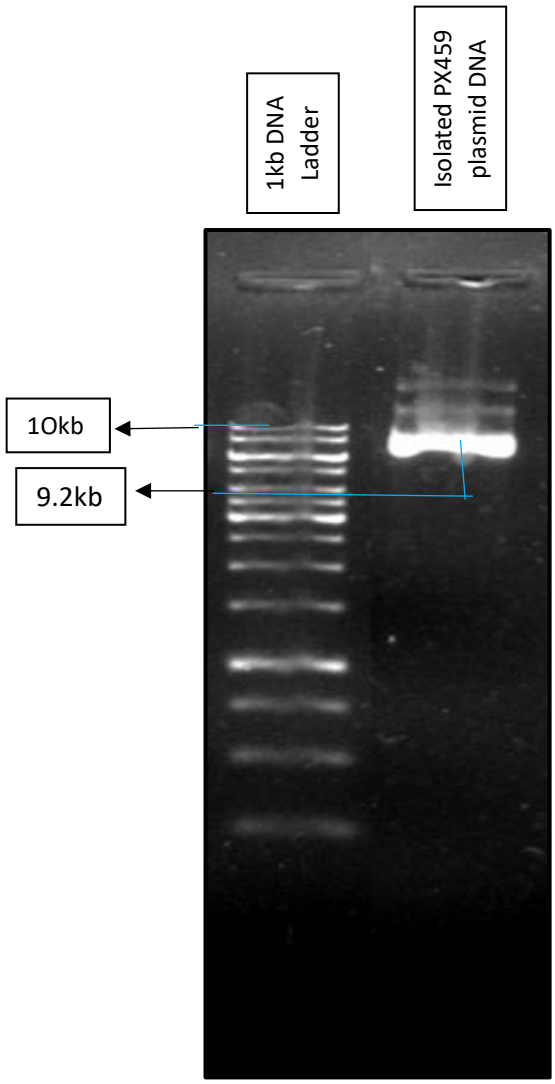

Fig.04 Visualization of Isolated Plasmid DNA in 1% agarose gel.

5. Confirmation of PX459 Plasmid by Restriction Digestion

After confirming the PX459 plasmid based on its size, this experiment was done to confirm the plasmid based on presence of restriction site. We knew based on the plasmid map that the PX459 plasmid has two EcoRI restriction sites present at 5077 and 5746 position. The EcoRI is a type II restriction endonuclease which recognizes the 5-'GAATTC-3' sequence and cleaves the DNA strands at the same site generating sticky ends, so we conducted the EcoRI restriction digestion following our protocol and visualized it by running 1% agarose gel electrophoresis method. For comparison we ran 1 kb DNA ladder and undigested PX459 plasmid DNA along with our EcoRI digested DNA. Based on the presence of EcoRI cut sites in PX459 (5077,5746)

and the total number of base pairs of PX459 plasmid (9174) we calculated the possible position of the bands at – 8.2 kb and 688 bp. After running in 1.5% agarose gel, we visualized the gel and under UV and found the bands at the same position as our calculation suggested and no undigested or partial digested bands were seen, confirming a proper EcoRI digestion and the presence of PX459 in our isolated plasmid DNA.

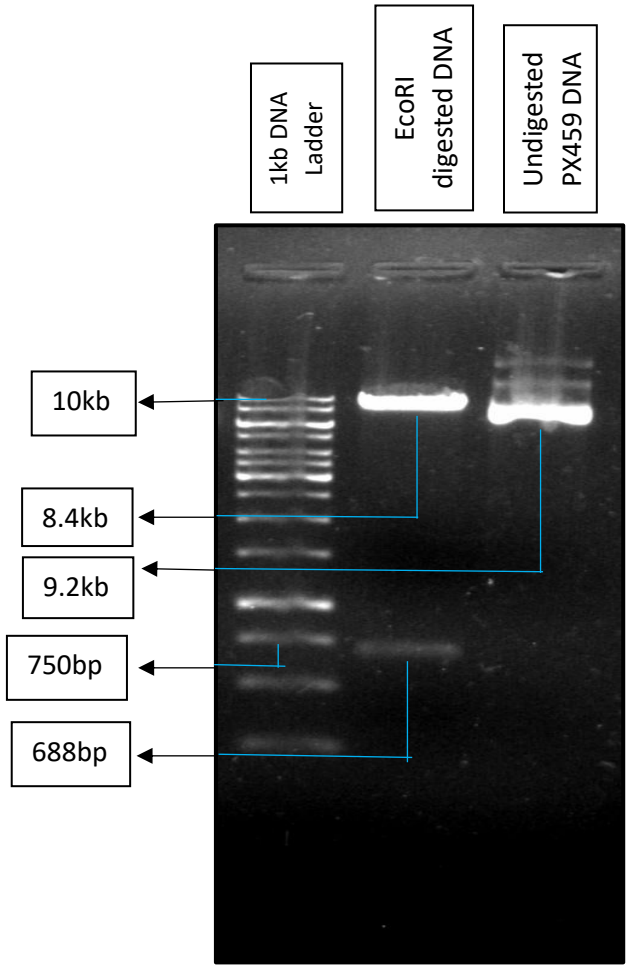

Fig.05 Visualization of EcoRI digested PX459 DNA.

6. BbsI Digestion of PX459 Plasmid and Purification of DNA

The PX459 has two BbsI restriction sites at 8977 and 8999 position, BbsI restriction endonuclease is a type IIS restriction enzyme and cleaves 2 bases downstream on the top strand and 6 bases downstream on the bottom strand of its recognition site (GAAGAC), creating a 4 base 5' overhang. We used BbsI to digest our PX459 plasmid following our standardized

protocol which was a crucial step, as the BbsI digestion creates 5' overhangs compatible with the sticky – CAAC – and – AAAC – ends of our sgRNA. This ensures proper binding and ensures the oligomers to be directionally cloned into the plasmid in our following downstream ligation experiment. Based on the presence of BbsI cut sites in PX459 (8977, 8999) and the total number of base pairs of PX459 plasmid (9174) we calculated the possible position of the bands at – 9.1 kb and 22bp. The 22bp band was too small to be detected by 1.5 % agarose gel and thus after running the agarose gel and visualizing it under UV, we got one crisp band at our desired position and no undigested or partial digested bands were seen. For size confirmation we ran 1kb DNA ladder and undigested PX459 DNA along with our BbsI digested DNA. After this we wanted to purify the digested DNA from the BbsI enzyme as it may hinder the downstream processes. We purified our digested DNA using SLS PCR Purification kit and then checked the DNA concentration using a nanodrop spectrophotometer (16 ng/µl).

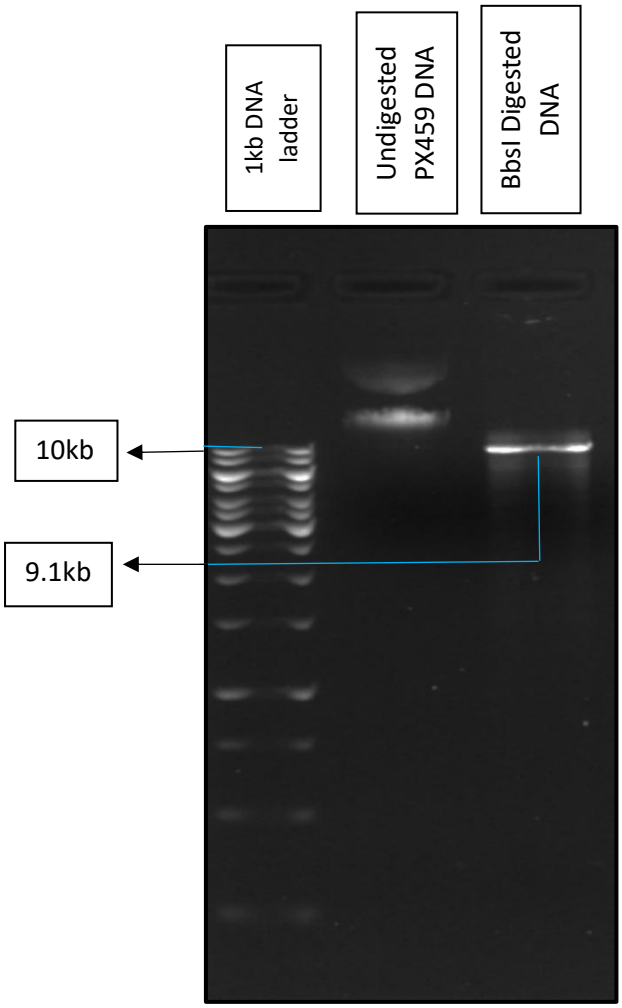

Fig.06: Visualization of BbsI digested PX459 DNA.

7. sgRNA Ligation and Transformation into Competent DH5α cells

The BbsI digestion of the plasmid created 5' overhangs in the PX459 plasmid which is compatible with the sticky – CAAC – and – AAAC – ends of our designed sgRNA. We set up two different sets of ligation mixture 'L1' and 'L2' and conducted the experiment following our standardized protocol to insert our designed sgRNA into the desired cloning site of PX459. After ligation we followed our standardized transformation protocol to transform competent DH5α cells with our recombinant plasmid PX459 plasmid, preparing recombinant clones. Once the transformation completed, we spread plated both sets of the recombinant clones on LB + amp plate. Ampicillin acts as a selective marker and after incubating the plate overnight we got colonies of recombinant clones with our sgRNA ligated into the PX459 plasmid backbone, confirming successful ligation and transformation. For comparison we simultaneously plated normal DH5α cells on a LB + amp plate as negative control. Next, we selected four recombinant clones from each set, resuspended it in 10µl NFW. Then we aliquoted 5µl and plated it on LB + amp plate in a checkerboard manner for downstream experimental use.

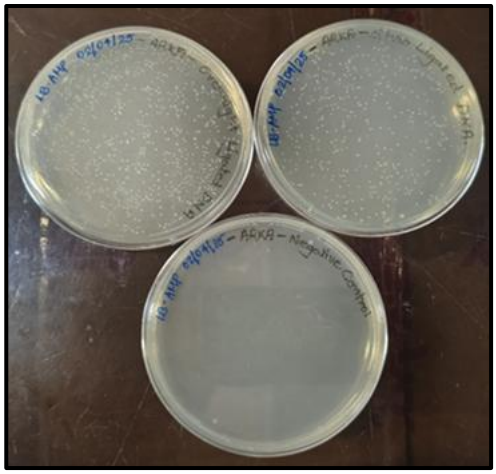

Fig.07: Recombinant Clones and Negative Control

Fig.08: Ligated Colonies Replica Plated

8. Ligated Colonies Confirmation by Colony PCR and Isolation of Recombinant Plasmid DNA

After resuspending the selected ligated colonies in NFW 5µl of each colonies were aliquoted and mixed with PCR reaction mixture and subjected to insert specific colony PCR, following our standardized protocol. We set up total 9 PCR reaction in which 8 were for the selected colonies from 'L1' and 'L2' sets and in the last mixture was a negative control in which no colonies were added, this was set to check if there were any conntaminats present in our reaction mixture. Once the PCR cycles completed ran them in 1% agarose gel. We calculated our amplicon size (251bp) before visualization of the gel using the established formula –

Amplicon Size = (End position of reverse primer – Start position of forward primer) – 1

After this we visualised the gel and compared the band size with 1kb DNA ladder and confirmed successful ligation of our designed sgRNA in DH5α recombinant clones and no band in the negative control PCR mixture confirmed a contamination free reaction mixture. After confirming, we isolated plasmid DNA from the 8 recombinant clones by following our standardized plasmid DNA isolation protocol and checked DNA concentration under nanodrop spectrophotometer. Plasmid DNA from two recombinant clones with highes DNA concentration BRCA1ADE10.1 clone, BRCA1ADE10.2 clone were used to continue our experiments.

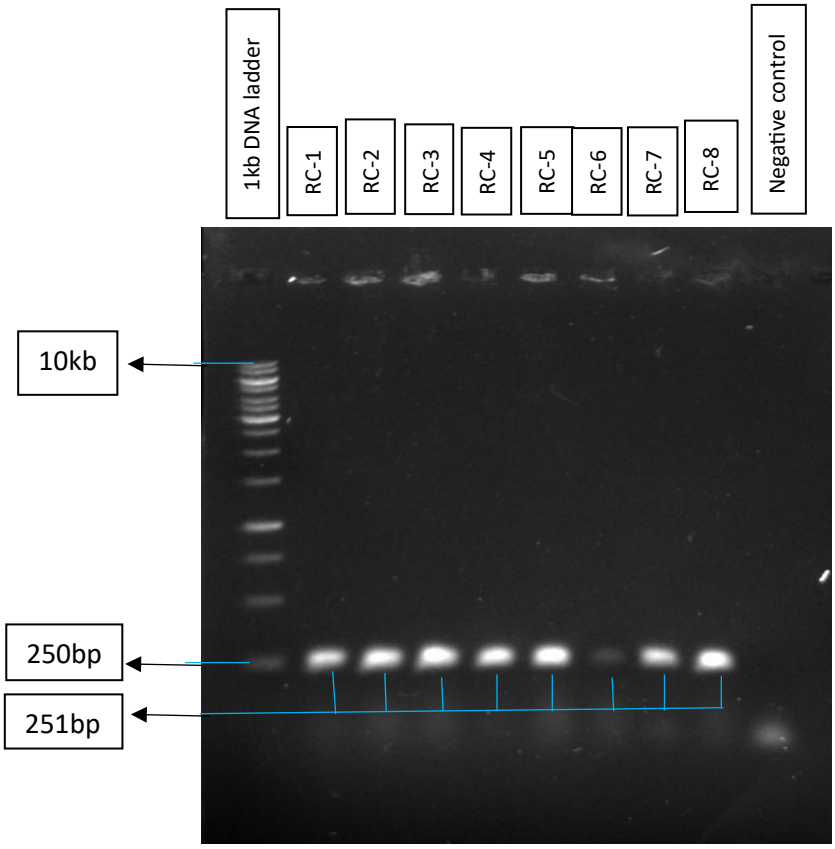

Fig.09: Visualization of Ligated Colonies by Colony PCR.

9. Vector Specific Reverse Primer Designing and PCR Confirmation

We designed a vector specific reverse primer to ensure our sgRNA got ligated into the PX459 Plasmid using bioinformatics resources like Addgene (https://www.addgene.org/62988/), NCBI Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast), and SnapGene (https://www.snapgene.com/). Once our designed reverse primer was delivered to us we calculated the amplicon size for the vector specific plasmid (404bp) and ran two end-point PCR using both insert specific and vector specific plasmid. We also set up a negative control PCR reaction to check for contaminants and 1kb DNA ladder for size confirmation. After the completion of both PCR cycles, we ran a 1% gel electrophoresis and visualised the gel and confirmed both amplicon sizes. The result confirmed a successful ligation and we compared both amplicon sizes.

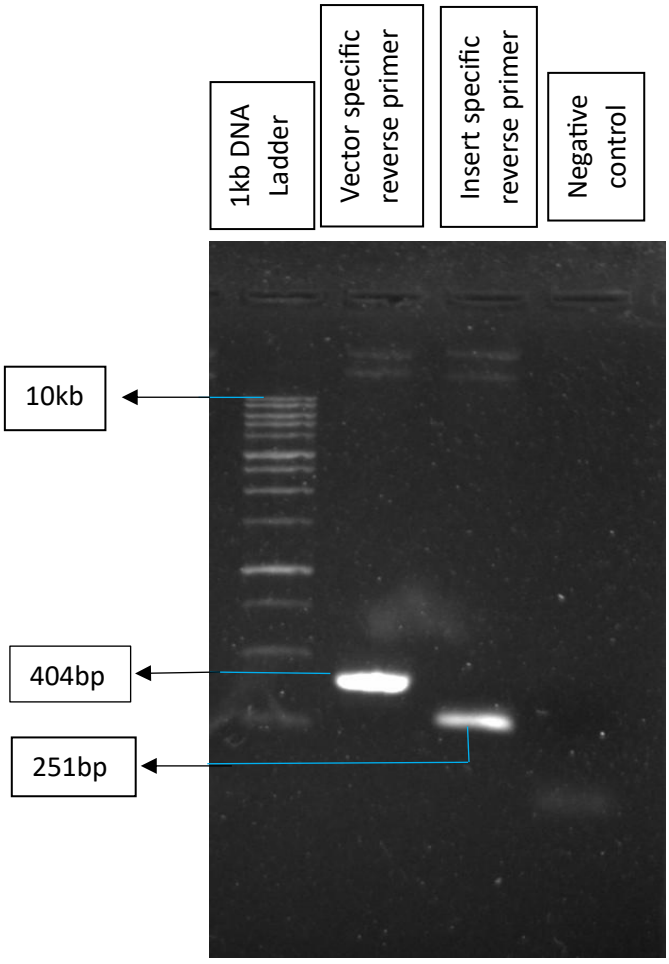

Fig.10: Visualization of Ligated Vector Specific Primer and Insert Specific Primer.

10.Sanger's Sequencing Confirmation of Recombinant Clones

After confirming presense of both vector and insert in our recombinant clones, we sequenced two of our recombinant clones with highest DNA Concentration -BRCA1ADE10.1 clone, BRCA1ADE10.2 clone and by analyzing the electropherograms we confirmed the location of our sgRNA top strand sequence 5' CACCGGGAGTTGGTCTGAGTGACA 3' is ligated. In BRCA1ADE10.1 clone the ligation occurred between 220 – 243 bases and in BRCA1ADE10.2 clone the ligation occurred between 235 – 258 bases.

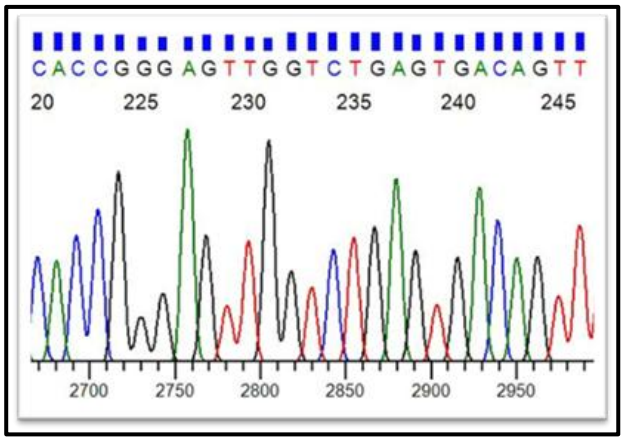

Fig.11: Ligation position of sgRNA in BRCA1ADE10.1 clone.

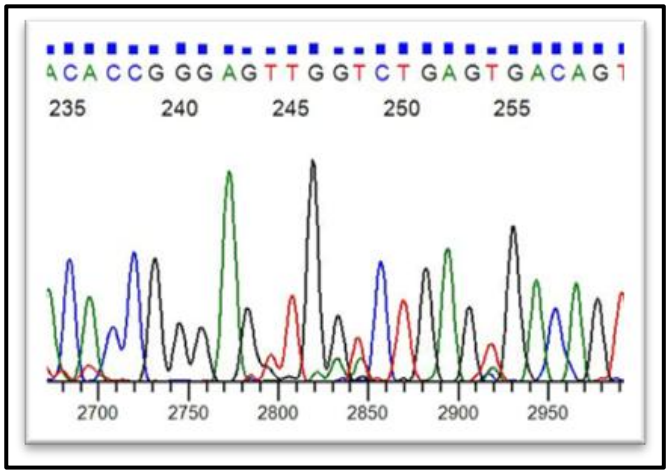

Fig.12: Ligation position of sgRNA in BRCA1ADE10.2 clone.

CONCLUSION

The present study focused on the construction of a single guide RNA (sgRNA) expression system specifically targeting the 10^{th} the largest exon of a critical tumor supressor gene BRCA1 using CRISPR-Cas9 technology. Mutations within the coding sequence especially within the 10^{th} exon is significantly associated with an increased risk of breast and ovarian cancers. Thus, our project work that focuses on the development of precise gene editing tools for such a vital genomic region is of considerable importance for both diagnostic and theraputic applications.

This research work successfully establishes a framework for designing,constructing, and validating sgRNAs targeting the exon 10 of BRCA1.

The study began with a detailed in-silico analysis to identify potential CRISPR target sites within the exon 10. Criterias such as on-target efficiency, off-target minimization,GC-content and secondary structure compatibility were considered during sgRNA design to ensure high accuracy and activity. Several sgRNA sequences were computationally evaluated using bioinformatic tools and based on the highest score the most effective sgRNA was chosen.

Based on in-silico validation, the selected sg-RNA sequence was cloned into a plasmid-based expression system under the control of a U6 promoter, ensuring a robust transcription of the sgRNA in mammalian cells. The plasmid construction was also verified through different restriction digestion and electrophoresis techniques. Finally, the recombinant clones were sequenced using Sanger's sequencing method and the electropherogram confirmed proper insertion and orientation of our designed sgRNA into the vector plasmid DNA.

The successful construction of the sgRNA expression system was an important milestone in the study and it contributes to the understanding of CRISPR-Cas9's precision and versatility when properly guided by a well designed sgRNAs. By targeting exon 10, a region which is highly relevant to cancer predisposition, this study lays the groundwork for future thereputic gene editing approaches aimed at correcting pathogenic mutations in BRCA1. This projectwork also emphasizes the importance of meticulous sgRNA design, which directly affects editing efficiency and specificity .

This project further highlights the potential applications of CRISPR-based technologies not only in gene editing but also in functional genomics studies. The sgRNA system developed here can be used to generate knock-out or knock-in models to study the functional consequences of BRCA1 mutations. This is particularly useful in preclinical cancer research, where understanding gene function at the molecular level can guide the development of new diagnostic markers or targeted therapies.

Nevertheless, this study acknowledges its several limitations and more comprehensive analysis such as deep-sequencing, off-target profiling and protein expression assessments are necessary to fully evaluate the efficacy and safety of the constructed system. Future refinements could include the use of high-fidelity Cas9 variants or chemically modified sgRNAs to further reduce the potential for off-target activity.

In conclusion, this thesis achieved its central objective of construction of a sgRNA expression system specifically targeting exon 10 of the BRCA1 gene using CRISPR-Cas9 technology and lays a solid foundation for further functional genomics research and therapeutic development. As CRISPR technology continues to evolve, such targeted systems will be indispensable in precision medicine, offering hope for individualized interventions in cancer and other genetic diseases and with continued innovation and validation, the future of genome editing in clinical oncology appears both promising and attainable.

APPENDIX

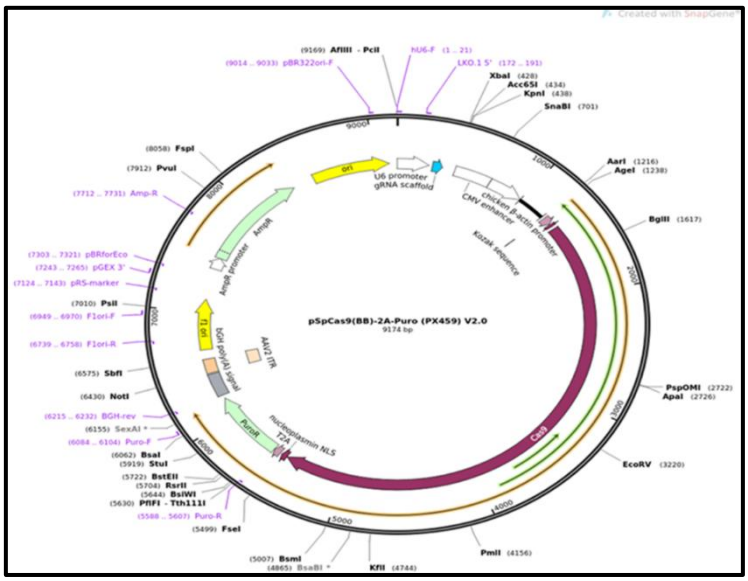

Fig.13: Plasmid Map of PX459 Plasmid.

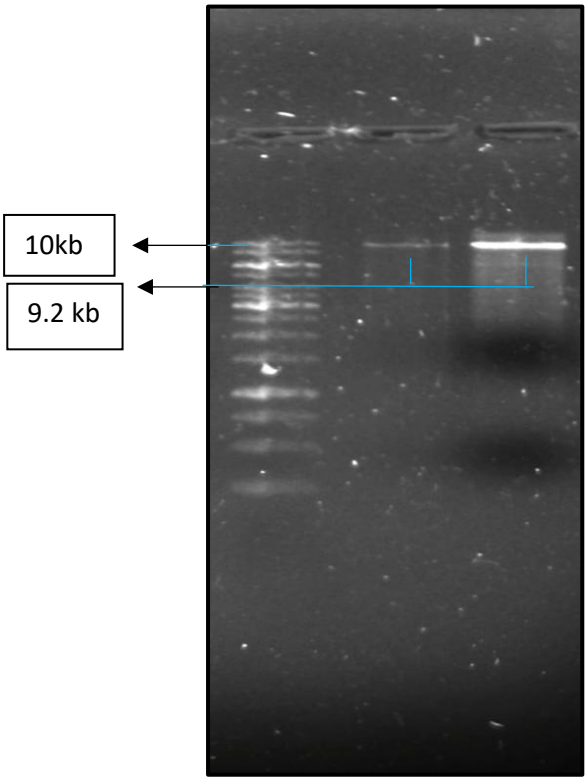

Fig.14: Confirmation of PCR Purified PX459 Plasmid DNA.

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- Addgene: CRISPR Guide
- https://www.addgene.org/crispr/zhang/
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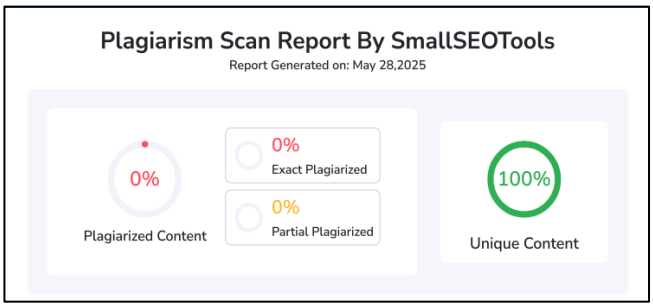
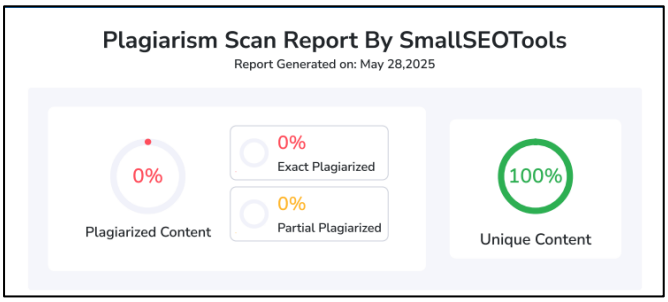
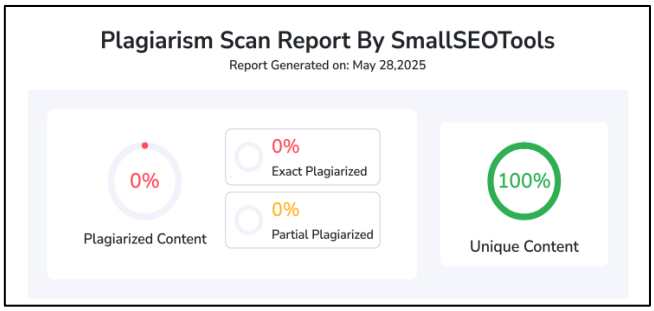


ABSTRACT



INTRODUCTION



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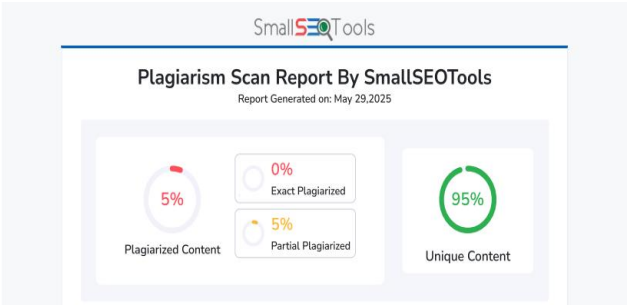
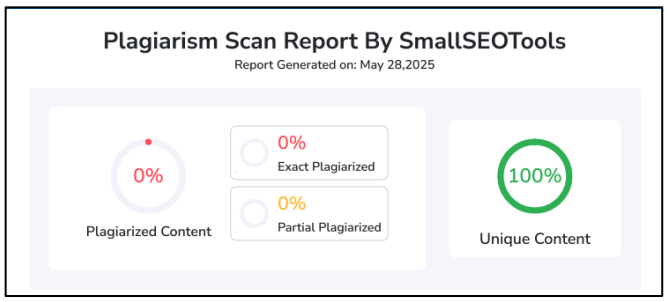
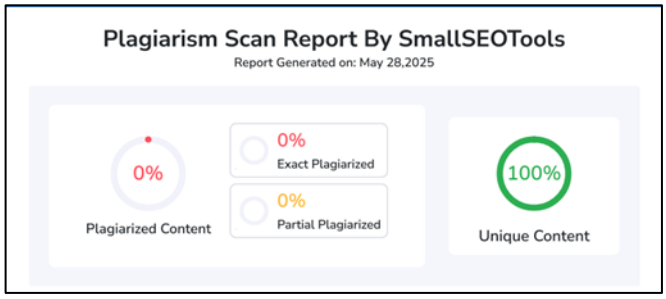


MATERIALS & METHODS



RESULT & DISCUSSION



CONCLUSION

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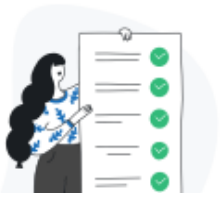
ABSTRACT

The BRCA1 gene plays an important role in maintaining the genomic stability and mutation of this gene increases the susceptibility towards breast and ovarian cancers. Targeted genome editing technologies like CRISPR-Cas9 have revolutionized genetic research through enabling precise genomic manipulation. The construction of a single -guide RNA (sgRNA) expression system targeting the BRCA1 gene represents a fundamental step towards elucidating gene function, modelling diseases and potentially developing gene therapies. In this study we have designed and constructed an sgRNA expression system specifically targeting 10th exon of the BRCA1 gene. Bioinformatic tools like NCBI, BLAST and CHOPCHOP were used to identify optimal sgRNA target sites with minimal off-target potential. The designed sgRNA was then cloned into a compatible expression vector, pSpCas9(BB)-2A-Puro (PX459). After successfully cloning the recombinant clones were confirmed through end-point PCR and Sanger's sequencing. This research highlights the accuracy and feasibility of constructing sgRNA expression system targeting the BRCA1 gene and provides foundation for functional genomics studies.

Keywords: BRCA1, CRISPR-Cas9, Gene editing, Restriction Digestion, Ligation, NCBI, Cloning, PX459, End point PCR, Sanger's Sequencing



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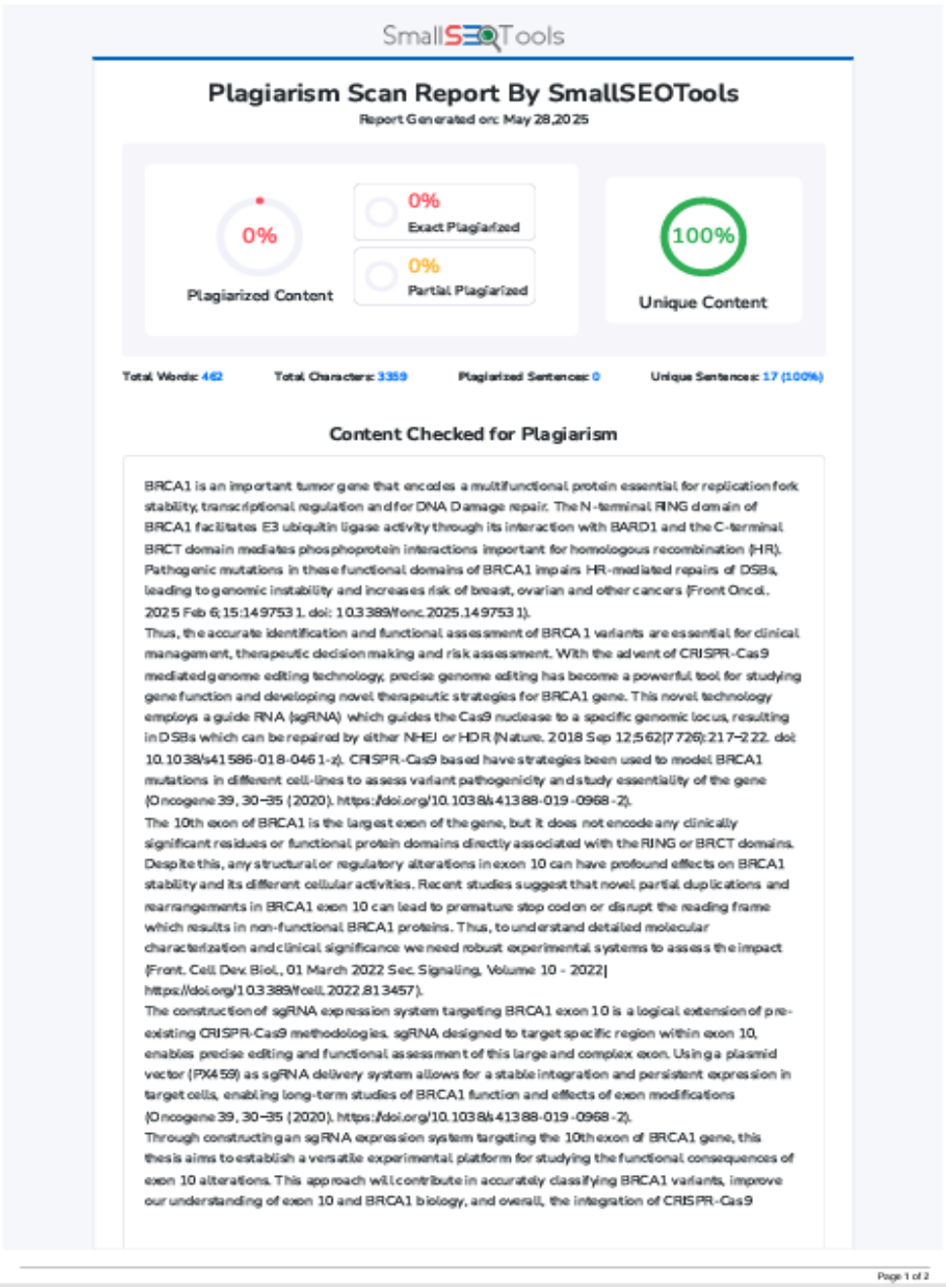
The Breast Cancer 1 gene or BRCA 1 gene is a crucial component of human genome which plays an important role in maintaining genomic stability. It is located on chromosome 17 at position q21 and consists 24 exons. BRCA1 codes for a tumor suppressor protein which plays crucial role for DNA damage repair and also maintains other cellular process. BRCA1 along with other repair proteins forms a multi-protein complex known as the BRCA1-associated genome surveillance complex or BASC, which operates the repair mechanism of double-strand DNA breaks (DSB) in a highly accurate process called Homologous Recombination (HR). In case of a normal human when a DNA damage occurs, BRCA1 gets recruited to the break site where it helps to recognize the damage and facilitates end resection by creating single-stranded DNA overhangs. It then assists in recruiting and stabilizing RAD51 which is a key recombinase and uses a sister chromatid as a template and accurately repairs the damaged DNA. Through this BRCA1 maintains the genomic stability and prevents the accumulation of genetic mutation that can potentially lead to uncontrolled cell division and cancer. But in case of inherited or acquired mutations in a BRCA1 gene, it loses its ability to participate in Homologous Recombination. This loss of function in BRCA1 leads the cells with dysfunctional BRCA1 gene to use error-prone DNA repair pathways such as non-homologous end joining (NHEJ) which frequently introduces mutations at DNA breakage sites. These mutations get accumulated in the cells and overtime, this accumulation of genetic alterations disrupts other critical genes like oncogenes and tumor suppressors, which leads to uncontrolled cell division and tumor development ultimately leading to cancer. BRCA1-related cancers often exhibit aggressive clinical features, including early onset and poor differentiation and women with BRCA1 mutation have a 65% lifetime risk of developing breast cancer and 39% risk of ovarian cancer

CRISPR-Cas9 technology, a novel and robust genome editing tool can be strategically employed to overcome these fatal effects of BRCA1 mutations by selectively knocking out mutated alleles which produces a dysfunctional BRCA1 protein. Though in nature it acts as a bacterial adaptive immunity response system, the Clustered Regularly Interspaced Short Palindromic Repeats or the CRISPR-Cas9 system is an RNA mediated system which is now used by the scientists to guide the Cas9 endonuclease to target DNA site and generate indels.

For an effective CRISPR mediated knockout the sgRNA has to be effectively designed as it controls the specificity, efficiency and outcome of the CRISPR mechanism. The single guide RNA (sgRNA) has two domains fused together, CRISPR RNA (crRNA) a 20-nucleotide sequence and the trans-activating CRISPR RNA (tracrRNA). This single RNA molecule binds with the Cas9 endonuclease and guides it to the precise target DNA sequence located immediately upstream of a PAM (Protospacer Adjacent Motif) sequence. Cas9 binds to the target sequence and it induces a double-strand break at the target site. The cell then repairs the break, through non-homologous end joining (NHEJ) which often introduces insertions or deletions (indels), that disrupts the reading frame and effectively inactivating the mutant allele. Enabling gene disruption, repair or replacement of the mutated gene. (Cureus. 2024



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BRCA1 is an important tumor gene that encodes a multifunctional protein essential for replication fork stability, transcriptional regulation and for DNA Damage repair. The N-terminal RING domain of BRCA1 facilitates E3 ubiquitin ligase activity through its interaction with BARD1 and the C-terminal BRCT domain mediates phosphoprotein interactions important for homologous recombination (HR). Pathogenic mutations in these functional domains of BRCA1 impairs HR-mediated repairs of DSBs, leading to genomic instability and increases risk of breast, ovarian and other cancers (Front Oncol. 2025 Feb 6;15:1497531. doi: 10.3389/fonc.2025.1497531).

Thus, the accurate identification and functional assessment of BRCA1 variants are essential for clinical management, therapeutic decision making and risk assessment. With the advent of CRISPR-Cas9 mediated genome editing technology, precise genome editing has become a powerful tool for studying gene function and developing novel therapeutic strategies for BRCA1 gene. This novel technology employs a guide RNA (sgRNA) which guides the Cas9 nuclease to a specific genomic locus, resulting in DSBs which can be repaired by either NHEJ or HDR (Nature. 2018 Sep 12;562(7726):217–222. doi: 10.1038/s41586-018-0461-z). CRISPR-Cas9 based have strategies been used to model BRCA1 mutations in different cell-lines to assess variant pathogenicity and study essentiality of the gene (Oncogene 39, 30–35 (2020). https://doi.org/10.1038/s41388-019-0968-2).

The 10th exon of BRCA1 is the largest exon of the gene, but it does not encode any clinically significant residues or functional protein domains directly associated with the RING or BRCT domains. Despite this, any structural or regulatory alterations in exon 10 can have profound effects on BRCA1 stability and its different cellular activities. Recent studies suggest that novel partial duplications and rearrangements in BRCA1 exon 10 can lead to premature stop codon or disrupt the reading frame which results in non-functional BRCA1 proteins. Thus, to understand detailed molecular characterization and clinical significance we need robust experimental systems to assess the impact (Front. Cell Dev. Biol., 01 March 2022 Sec. Signaling, Volume 10 - 2022| https://doi.org/10.3389/fcell.2022.813457).

The construction of sgRNA expression system targeting BRCA1 exon 10 is a logical extension of pre-existing CRISPR-Cas9 methodologies. sgRNA designed to target specific region within exon 10, enables precise editing and functional assessment of this large and complex exon. Using a plasmid vector (PX459) as sgRNA delivery system allows for a stable integration and persistent expression in target cells, enabling long-term studies of BRCA1 function and effects of exon modifications (Oncogene 39, 30–35 (2020). https://doi.org/10.1038/s41388-019-0968-2).

Through constructing an sgRNA expression system targeting the 10th exon of BRCA1 gene, this thesis aims to establish a versatile experimental platform for studying the functional consequences of exon 10 alterations. This approach will contribute in accurately classifying BRCA1 variants, improve our understanding of exon 10 and BRCA1 biology, and overall, the integration of CRISPR-Cas9

Page 1 of 2

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Primarily we need to design an efficient single guide RNA for targeting exon 10 of BRCA1 gene with the Px459 Cas 9 vector. The sgRNA designing is an important step for CRISPR-Cas9 mediated knockout. In order to do that an online software CHOPCHOP (https://chopchop.cbu.uib.no) was used and the sgRNA sequences were generated by us following the steps mentioned below.
Firstly, NCBI (https://www.ncbi.nlm.nih.gov) portal was opened. Next, BRCA1 gene was searched. Next, in 'GenBank' option we searched and selected our desired TranscriptId (NM_007294.4) of the BRCA1 gene. After selecting our desired TranscriptId, our desired exon (Exon 10) was searched and the sequence was copied. Next, the copied exon sequence was pasted in CHOPCHOP and specific parameters for the designed sgRNA were set.
For 'Target specific region of the gene' option 'Coding Region' was selected. For 'Restrict targeting' option 'Search exons and Immediate short flanking regions' was selected. In the 'Isoform consensus determined by' option 'Intersection' option was selected. In 'Pre-filtering' option the 'Minimum required GC (%) content' was set between Minimum: 40% and Maximum:60%. Based on our given parameters the software generated multiple sequences. After comparing the efficiency, GC content, self-complementarity and other parameters our desired top and bottom sgRNA sequences were selected and ordered from Syngynx.
After the designed sgRNAs were delivered to us we needed to anneal the oligomers in order use it in our downstream experiments. To anneal our oligomers first, we ran an empty spin to collect the lyophilized oligomers in the bottom of the test vials. Next, nuclease free water was added in the vials to dilute the lyophilized oligomers. 308.5µl of nuclease free water was added to the top strand oligomer and 313.5µl of nuclease free water was added to the bottom strand of the oligomer to attain 100Mm concentration and another quick spin was performed for proper mixing. Next a reaction mixture was set up by using the components mentioned below in (Table 4.2.1).

After a successful transformation of the plasmid into the bacterial cells, a volume of 10 ml of LB + ampicillin growth medium was prepared in two separate 50 ml centrifuge tubes. Next, a single colony was isolated for each tube from the replica plate and added to our LB + ampicillin culture. This culture was then put in the shaker-incubator for bacterial growth at 37˚C and 130 rpm. After 16 hours of incubation, the tubes were taken out of the shaker-incubator, and 4.5 ml volume of the cell culture was aliquoted into three microcentrifuge tubes (1.5 ml each) and centrifuged at 1000 rpm for 3 minutes. After the first centrifugation, the supernatant was discarded carefully using a micropipette. The remaining 4.5 ml volume of the cell culture was then aliquoted into the same centrifuge tubes and centrifuged again for 3 minutes at 10000 rpm. The supernatants were then discarded, and the cell pellets were resuspended by adding 250µl of P1 buffer (Resuspension buffer). The mixture was aspirated carefully until it turned cloudy with no visible cell clumps. Next, 250µl volume of P2 buffer (Lysis buffer) solution was added into each tube and was gently inverted mixed for 2 minutes.

Immediately after 2 minutes of inverted mixing, 350µl of PN-3 buffer (Neutralizing buffer) was added to the solution, and the solution was gently inverted and mixed for 2-3 minutes. Next, the tubes were centrifuged at 10000 rpm for 10 minutes. Two distinct layers were noticed inside the microcentrifuge tube at this stage. The clear supernatant was separated from three tubes and added to a single spin column using a micropipette and centrifuged at 10000 rpm for 1 minute. Once the centrifugation was done, the flowthrough was discarded. This step was repeated until the entire supernatant from three tubes was spun down in a single spin column. After this, we added 500µl of PW-1 wash buffer

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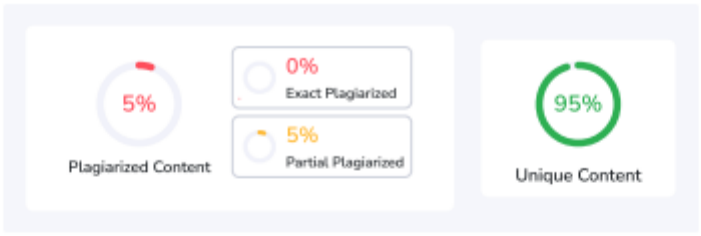

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This was a confirmatory step to ensure that the isolated plasmid DNA was indeed PX459. For this test we took a 100µl microcentrifugation tube and prepared a reaction mixture using the components given below in (Table 4.9.1). The reaction mixture was incubated for 2 hours in the water-bath set at a temperature at 37˚C. After 2 hours incubation we visualized the DNA sample using 1.5% agarose gel and by compared the fragment size with a 1kb ladder and an undigested DNA sample of PX459 plasmid.

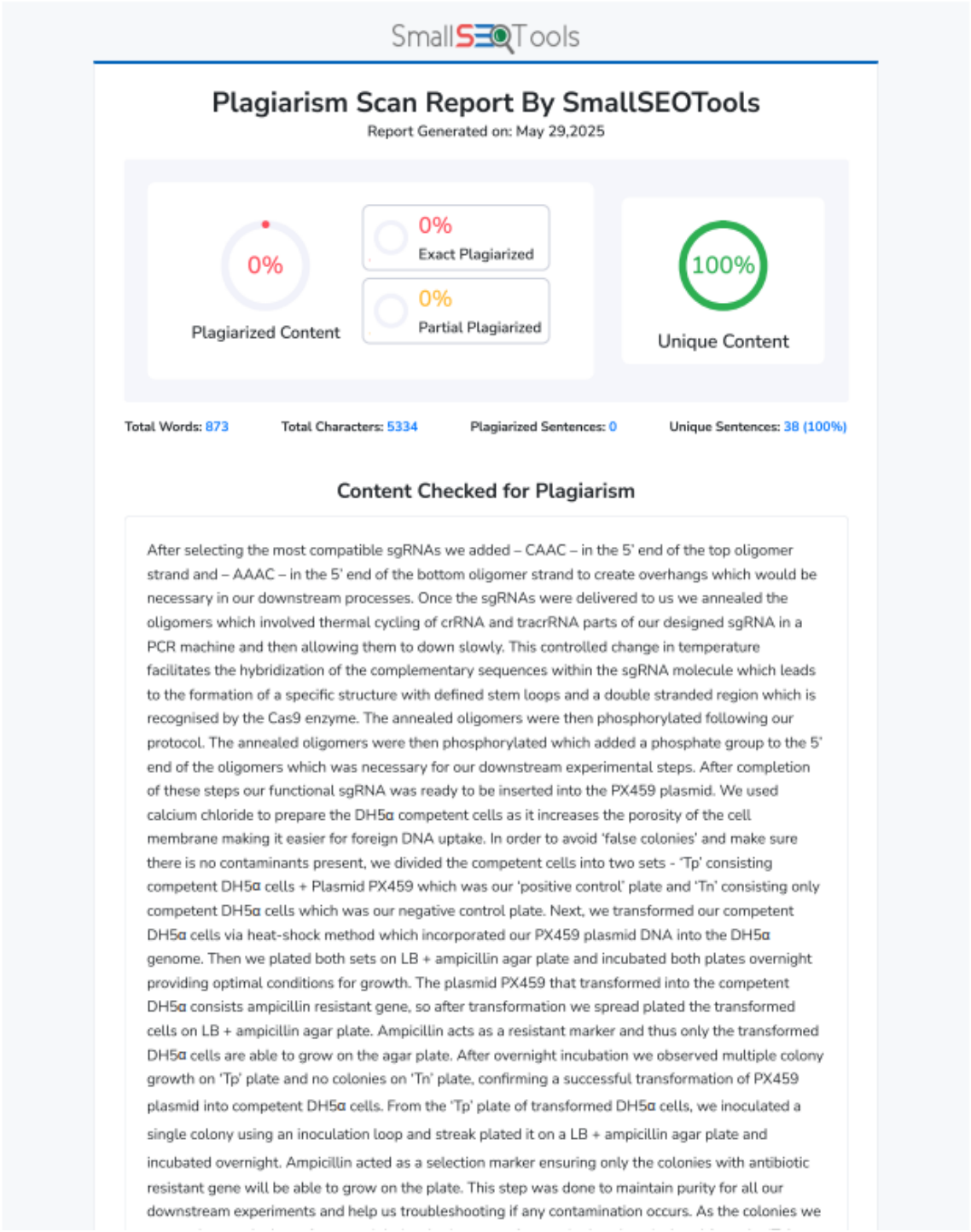
After confirming presence of PX459 plasmid through EcoRI restriction digestion, we treated our plasmid with BbsI restriction endonuclease. The reaction mixture was set up by using the components mentioned below in (Table 4.10.1).

This reaction mixture was incubated for 2 hours in the water-bath set at a temperature of 37˚C. We visualized the DNA sample using 1.5% agarose gel and by comparing the fragment size with a 1kb ladder and an undigested DNA sample we confirmed the restriction digestion. After this in order to remove any unwanted restriction enzyme present, we purified the BbsI Digested DNA using SLS PCR purification kit. For purification we first aliquoted our Bbs1 digested DNA into a spin column and added PW1 buffer in it. Then we centrifuged the column at 10,000 rpm for 1 minute and discarded the flowthrough. Next, we added PW2 into the spin column and again centrifuged the column at 10,000 rpm for 1 minute and discarded the flowthrough and performed an empty spin to eliminate any excess wash buffer. Finally, we eluted our purified PX459 using 50µl pre-heated nuclease free water.

After purifying our digested plasmid DNA in order to ligate our designed annealed sgRNA oligomers into the plasmid DNA we setup two sets of ligation reaction named L1 and L2. The L1 set was incubated at 16˚C for overnight and the L2 set was incubated at room temperature for 6 hours. The reaction mixture was prepared in a 100µl microcentrifuge tube using the components mentioned below in (Table 4.11.1) and (Table 4.11.2). A negative control was also set up which contained all the mentioned components except the annealed phosphorylated oligomers.

This was a confirmation step to ensure the proper ligation of our designed guide RNA into our plasmid vector PX459. We set up our reaction mixture (Table 4.13.1) using an insert specific primer (BRCA1 bottom oligomer) for 10 PCR tubes with 20 µl reaction mixture volume for each tube with the reaction components mentioned below. Next, we added 5µl of each resuspended colonies in separate PCR tubes and ran a colony PCR (Table 4.13.2). After the PCR we confirmed the ligation by running the PCR mixture in 1.5% agarose gel.After confirmation we isolated plasmid DNA from our ligated colonies using our standardized plasmid DNA isolation protocol and measured the concentration of isolated DNA using nanodrop spectrophotometer. Plasmid DNA from two recombinant clones with highest DNA concentration BRCA1ADE10.1 clone (176.4 ng/µl) and BRCA1ADE10.2 clone (104.7 ng/µl) were used to continue the downstream experiments. For further confirmation of the ligation, we also designed a 18-25 nucleotide long vector specific reverse primer to run an end point PCR

simultaneous to our insert specific primer end point PCR for comparison. For designing the reverse primer, first we opened SnapGene software (https://www.snapgene.com) and copied FASTA sequence 150bp away upstream and downstream from the BbsI cut sites. Next, we opened NCBI Primer BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primerblast) set parameters (Tm, GC Content and minimal secondary structures) and pasted the FASTA sequence. The NCBI Primer BLAST provided multiple primer sequence from which we selected the most compatible primer (Table 4.14.1) and ordered it.After designing our vector specific we set up two PCR reaction mixture 'P1' and 'P2' where 'P1' contained the insert specific primer and the 'P2' contained the vector specific primer. We set our reaction with the reaction components mentioned below and ran end point PCR following the cycles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After selecting the most compatible sgRNAs we added – CAAC – in the 5' end of the top oligomer strand and – AAAC – in the 5' end of the bottom oligomer strand to create overhangs which would be necessary in our downstream processes. Once the sgRNAs were delivered to us we annealed the oligomers which involved thermal cycling of crRNA and tracrRNA parts of our designed sgRNA in a PCR machine and then allowing them to down slowly. This controlled change in temperature facilitates the hybridization of the complementary sequences within the sgRNA molecule which leads to the formation of a specific structure with defined stem loops and a double stranded region which is recognised by the Cas9 enzyme. The annealed oligomers were then phosphorylated following our protocol. The annealed oligomers were then phosphorylated which added a phosphate group to the 5' end of the oligomers which was necessary for our downstream experimental steps. After completion of these steps our functional sgRNA was ready to be inserted into the PX459 plasmid. We used calcium chloride to prepare the DH5α competent cells as it increases the porosity of the cell membrane making it easier for foreign DNA uptake. In order to avoid 'false colonies' and make sure there is no contaminants present, we divided the competent cells into two sets - 'Tp' consisting competent DH5α cells + Plasmid PX459 which was our 'positive control' plate and 'Tn' consisting only competent DH5α cells which was our negative control plate. Next, we transformed our competent DH5α cells via heat-shock method which incorporated our PX459 plasmid DNA into the DH5α genome. Then we plated both sets on LB + ampicillin agar plate and incubated both plates overnight providing optimal conditions for growth. The plasmid PX459 that transformed into the competent DH5α consists ampicillin resistant gene, so after transformation we spread plated the transformed cells on LB + ampicillin agar plate. Ampicillin acts as a resistant marker and thus only the transformed DH5α cells are able to grow on the agar plate. After overnight incubation we observed multiple colony growth on 'Tp' plate and no colonies on 'Tn' plate, confirming a successful transformation of PX459 plasmid into competent DH5α cells. From the 'Tp' plate of transformed DH5α cells, we inoculated a single colony using an inoculation loop and streak plated it on a LB + ampicillin agar plate and incubated overnight. Ampicillin acted as a selection marker ensuring only the colonies with antibiotic resistant gene will be able to grow on the plate. This step was done to maintain purity for all our downstream experiments and help us troubleshooting if any contamination occurs. As the colonies we

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The PX459 has two BbsI restriction sites at 8977 and 8999 position, BbsI restriction endonuclease is a type IIS restriction enzyme and cleaves 2 bases downstream on the top strand and 6 bases downstream on the bottom strand of its recognition site (GAAGAC), creating a 4 base 5' overhang. We used BbsI to digest our PX459 plasmid following our standardized protocol which was a crucial step, as the BbsI digestion creates 5' overhangs compatible with the sticky – CAAC – and – AAAC – ends of our sgRNA. This ensures proper binding and ensures the oligomers to be directionally cloned into the plasmid in our following downstream ligation experiment. Based on the presence of BbsI cut sites in PX459 (8977, 8999) and the total number of base pairs of PX459 plasmid (9174) we calculated the possible position of the bands at – 9.1 kb and 22bp. The 22bp band was too small to be detected by 1.5 % agarose gel and thus after running the agarose gel and visualizing it under UV, we got one crisp band at our desired position and no undigested or partial digested bands were seen. For size confirmation we ran 1kb DNA ladder and undigested PX459 DNA along with our BbsI digested DNA. After this we wanted to purify the digested DNA from the BbsI enzyme as it may hinder the downstream processes. We purified our digested DNA using SLS PCR Purification kit and then checked the DNA concentration using a nanodrop spectrophotometer (16 ng/µl).

The BbsI digestion of the plasmid created 5' overhangs in the PX459 plasmid which is compatible with the sticky – CAAC – and – AAAC – ends of our designed sgRNA. We set up two different sets of ligation mixture 'L1' and 'L2' and conducted the experiment following our standardized protocol to insert our designed sgRNA into the desired cloning site of PX459. After ligation we followed our standardized transformation protocol to transform competent DH5α cells with our recombinant plasmid PX459 plasmid, preparing recombinant clones. Once the transformation completed, we spread plated both sets of the recombinant clones on LB + amp plate. Ampicillin acts as a selective marker and after incubating the plate overnight we got colonies of recombinant clones with our sgRNA ligated into the PX459 plasmid backbone, confirming successful ligation and transformation. For comparison we simultaneously plated normal DH5α cells on a LB + amp plate as negative control. Next, we selected four recombinant clones from each set, resuspended it in 10µl NFW. Then we aliquoted 5µl and plated it on LB + amp plate in a checkerboard manner for downstream experimental use. After resuspending the selected ligated colonies in NFW 5µl of each colonies were aliquoted and mixed with PCR reaction mixture and subjected to insert specific colony PCR, following our standardized protocol. We set up total 9 PCR reaction in which 8 were for the selected colonies from 'L1' and 'L2' sets and in the last mixture was a negative control in which no colonies were added, this was set to check if there were any conntaminats present in our reaction mixture. Once the PCR cycles completed ran them in 1% agarose gel. We calculated our amplicon size (251bp) before visualization of the gel using the established formula –

Amplicon Size = (End position of reverse primer – Start position of forward primer) – 1

After this we visualised the gel and compared the band size with 1kb DNA ladder and confirmed

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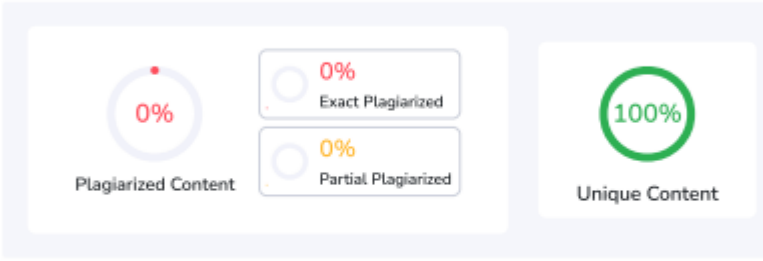

Total Words: 527 Total Characters: 3763 Plagiarized Sentences: 0 Unique Sentences: 22 (100%)

Content Checked for Plagiarism

The present study focused on the construction of a single guide RNA (sgRNA) expression system specifically targeting the 10th the largest exon of a critical tumor supressor gene BRCA1 using CRISPR-Cas9 technology. Mutations within the coding sequence especially within the 10th exon is significantly associated with an increased risk of breast and ovarian cancers. Thus, our project work that focuses on the development of precise gene editing tools for such a vital genomic region is of considerable importance for both diagnostic and theraputic applications.
This research work successfully establishes a framework for designing,constructing, and validating sgRNAs targeting the exon 10 of BRCA1.
The study began with a detailed in-silico analysis to identify potential CRISPR target sites within the exon 10. Criterias such as on-target efficiency, off-target minimization,GC-content and secondary structure compatibility were considered during sgRNA design to ensure high accuracy and activity. Several sgRNA sequences were computationally evaluated using bioinformatic tools and based on the highest score the most effective sgRNA was chosen.
Based on in-silico validation, the selected sg-RNA sequence was cloned into a plasmid-based expression system under the control of a U6 promoter, ensuring a robust transcription of the sgRNA in mammalian cells. The plasmid construction was also verified through different restriction digestion and electrophoresis techniques. Finally, the recombinant clones were sequenced using Sanger's sequencing method and the electropherogram confirmed proper insertion and orientation of our designed sgRNA into the vector plasmid DNA.
The successful construction of the sgRNA expression system was an important milestone in the study and it contributes to the understanding of CRISPR-Cas9's precision and versatility when properly guided by a well designed sgRNAs. By targeting exon 10, a region which is highly relevant to cancer predisposition, this study lays the groundwork for future thereputic gene editing approaches aimed at correcting pathogenic mutations in BRCA1. This projectwork also emphasizes the importance of meticulous sgRNA design, which directly affects editing efficiency and specificity .

This project further highlights the potential applications of CRISPR-based technologies not only in gene editing but also in functional genomics studies. The sgRNA system developed here can be used to generate knock-out or knock-in models to study the functional consequences of BRCA1 mutations. This is particularly useful in preclinical cancer research, where understanding gene function at the molecular level can guide the development of new diagnostic markers or targeted therapies.
Nevertheless, this study acknowledges its several limitations and more comprehensive analysis such as deep-sequencing, off-target profiling and protein expression assessments are necessary to fully evaluate the efficacy and safety of the constructed system. Future refinements could include the use of high-fidelity Cas9 variants or chemically modified sgRNAs to further reduce the potential for off-target